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RESEARCH ARTICLE

Novel rapid in vitro cytotoxicity test on mammalian cells based on an electrochemical measuring method

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Abstract The proliferation of pharmaceuticals and chemicals and the increasing manufacture of medical goods have made an assessment of their potential toxicological risks to man and the environment indispensable. Hence, a wide range of tests has been developed in order to generate data on the harmful effects of chemicals and pharmaceuticals on organisms. Initially, toxicological data were commonly collected from tests involving animals; however, on account of ethical objections, they are gradually being replaced by in vitro cytotoxicity tests. In this work, a new in vitro screening method for the determination of cytotoxicity, based on a novel electrochemical bioactivity sensor system, was implemented. The evaluation was based on current–time curves of a potentiostatic measurement proportional to the reduction of mediator molecules by mammalian cells. Depending on the number of reduced mediator molecules used by the cells, a current signal is produced, which provides information about the viability of living cells. By adding a toxic test substance to the cell, a reduction in the current signal can be observed, depending on the cytotoxicity of the substance. It is possible to quickly create a specific cytotoxic curve and to determine the corresponding inhibitory concentrations (IC_{50} value). First tests were performed on three mammalian cell lines and eight model compounds using this electrochemical measurement system. The IC_{50} values obtained corresponded well with the toxicity determined with an established reference cytotoxicity assay (MTT test) and with data reported in the literature.

Keywords Bioelectrochemistry · Mediator · Cytotoxicity · IC50 · Cytotoxicity test · Toxicological risk · Bioactivity sensor

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1 Introduction

In the past few years, the European Union has introduced many new regulations to protect man and the environment from the impact of hazardous chemicals and pharmaceuticals. These regulations oblige industry to generate toxicological data on all chemicals on the market in order to determine their safety. Since the collection of toxicological data became mandatory, mainly animals have been used in experiments to assess the potential risks of chemicals to humans and other biota. The "acute oral toxicity test" (LD_{50} test) described in OECD (Organisation for Economic Co-operation and Development) Test Guideline 401 of 1987 [1] was mainly used for this purpose. Since 2002, the OECD has replaced this classical LD_{50} test by new modified methods described in three guidelines [2–5]. These methods still use animals, but the endpoint is not exclusively the death of the animal. In the last 20 years, efforts have been made to reduce the number of animal experiments. In this context, many new standardised protocols, mostly based on cell cultures, have been developed to assess potentially hazardous chemicals [6]. These methods ensure sound scientific applications in contrast to

unspecific animal tests. They generate reliable data for a critical assessment of human health hazards and risks in a shorter time period and at lower cost and substantially promote the "3Rs" (replacement, reduction and refinement) with respect to the use of animals. Currently, there are a number of methods and cytotoxicity tests, which represent a promising new approach in this field [7]. Although the first tests on cell cultures to determine the toxicity of DDT were performed before 1945 [8], the breakthrough only came with "The Multicenter Evaluation of In Vitro Cytotoxicity" study (MEIC), which was published in 1999 [9, 10]. In the MEIC study, 50 chemicals were tested using different in vitro toxicity assays. The similarity of the results indicated that simple assays with cell lines could replace complicated primary culture systems for many testing purposes [9–11]. The acceptance of in vitro cytotoxicity tests has increased in the last few years. For example, the "3T3 Neutral Red Uptake Phototoxicity Assay" (3T3 *NRU* PT) was incorporated into OECD guidelines in 2004 with the collaboration of Spielmann and Liebsch [12, 13]. This test is a prime example of the potential of this group of in vitro cytotoxicity assays to increasingly replace animal tests. Furthermore, the controversial "Draize Test" (ocular irritation testing), used since 1944 [14], triggered the quest for alternative toxicity assays and other cell-based systems [15–17]. An in vitro toxicological assay with mouse lymphoma cells and an in vitro 3t3 NRU phototoxicity assay have also been used successfully in the screening of environmental samples (liquid samples and soil samples) [18], and good correlations with in vivo results have been found [19].

A similar development can be traced in the field of ecotoxicology [20]. In ecotoxicological investigations, chemical analysis has been used as a standard method to detect pollutants in water, soil and air. Additionally, various bacteria, algae, plants, fish and amphibian models have been used to test for the toxicity of chemicals in the environment. New investigations presented in this field include an array technology that uses recombinant bioluminescent bacteria to detect and classify environmental toxicity [21] and an amperometric screen-printed algal biosensor for the detection of environmental toxic compounds [22]. In addition, some fish cell-based toxicity assays, including the cell lines RTgill-W1, R1, RTG-2 and others, showing good correlations with in vivo toxicity results, have also been developed [19, 23–26].

With respect to a measured endpoint, the most frequently described cytotoxic assays are based on photometric methods, which allow assessment of cell activity, cell growth or cell morphology. In addition, new approaches based on electrochemical techniques, allowing fast detection of cellular or microbial activity, easier handling and reliable data assessment, have been explored [27]. Examples in the literature describe systems permitting rapid activity measurement [28, 29] and electrochemically based fermentation control [30] as well as the detection of cell numbers [31, 32]. The use of electrochemical biosensors on a chip for the analysis of toxicological potential, including the measurement of multi-cell parameters using a three-dimensional cell culture [33] or real-time monitoring cytotoxicity system [34], has also been reported. In 2007, Ceriotti presented a further approach by combining different single-chip electrical measurement methods enabling the simultaneous monitoring of multi-parametric metabolic parameters [35]. The use of other single-cell organisms like the bacteria *Escherichia coli* [36, 37] or algae [38] in electrochemical biosensors for toxicity testing has also been demonstrated. Furthermore, electrochemical biosensors for the detection of specific compound classes, such as phenols or nitrophenols, showing good correlations with other published data [39, 40], have been developed.

The present paper demonstrates the further development of a fast and sensitive electrochemical response system [41, 42] to determine the activity and viability of mammalian cells. Validation of the system using three mammalian cell lines and eight model chemical compounds is presented.

2 Experimental

2.1 Overall design

The study presents the application of a novel amperometric sensor system featuring a device with four independent measurement cells. For the measurements, mammalian cell lines (CHO, 3t3, V79) were embedded in a gel containing a redox mediator, duroquinone and exposed to a range of concentrations of eight different chemicals that included model toxicants, pesticides and pharmaceuticals. Electrochemical measurements allowed the estimation of IC_{50} values, which were compared with the results of reference cytotoxicity tests run in parallel as well as with data from the literature.

2.2 Chemicals and media

2.2.1 Culture media

Cell culture media, sera and other chemicals were obtained from BIOCHROM, Berlin, Germany. CHO cells were cultivated in Ham's F12 [FG0815], 3t3 cells in Dulbeccós MEM [FG0435] and V79 in RPMI 1640 [FG1215]. In total, 10 % foetal bovine calf serum [S0115, batch 0410G], 1 % penicillin/streptomycin solution [A2213] and 1 % amphotericin B [A2612] were added to all media. The media were stored at 4 °C in a refrigerator for a maximum of 2 weeks.

2.2.2 Test substances used

The chemicals used were as follows: NaCl [Roth-Laborbedarf Germany, 9265.2], isoniazide [SIGMA, 13377], lindane [ALDRICH, 233390], paracetamol [SIGMA, A7302], acetylsalicylic acid (ASS) [SIGMA, A3160], trichlorfon [FLUKA, 45698], cyclohexamide [FLUKA, 46401] and paraquat [FLUKA, 36541].

2.3 Measuring principle

For the toxicological studies, an amperometric measurement system, consisting of a reference electrode, counter electrode and working electrode (three-electrode system), was used. The reaction of a metabolically reduced electron transfer mediator at the working electrode produces a current, which can be recorded by a potentiostat. For this, the mediator should reach the cell in its oxidised form to be available as an electron acceptor and should exhibit low toxicity to the cells. Mediators are applied as electroactive substances, acting as electron carriers between organisms and the electrochemical system. A working hypothesis is that the mediators compete with some redox systems in the metabolism [42], though, normally, oxygen is a better electron acceptor for the organism [46]. The mediator reacts rapidly in competition with the electron donors of the cells, quickly leaves the cells after the reaction and emits electrons at the electrode surface. A further advantage of the mediator is the high negative redox potential. This effect results in a high potential difference between the electrodes and this mediator [43–45]. The current measured is proportional to the concentration of the mediator. In the present study, the best mediator for mammalian cells was found to be duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone). In mammalian cells, ubiquinone is a key molecule in cellular metabolism, which accepts hydrogen from coenzymes as well as succinate and fatty acids and acts as an electron pool and electron transport molecule. The mediator duroquinone has structural similarities to ubiquinone and was well accepted by all cells tested (Fig. 1).

To increase the yield of electron transfer to the mediator molecules, it is necessary to minimise oxygen transfer to the cells. For the measurement process, the cells and mediator are immobilised in a Sephadex^{TM}-G200 superfine gel (see Fig. 2). The cells and the mediator are well distributed in the reaction vessel without stirring, the penetration of air (oxygen) into the gel is limited and the cells consume the oxygen dissolved in the original media. The gel structure can also enclose and fix powdery lipophilic mediators and lipophilic test substances without an additional solubiliser. This fulfils the requirement that the mediator should act as an acceptor of both electrons and protons in the cells [42] since oxygen is a better acceptor of electrons from the organism.

2.4 Evaluation method

The evaluation was based on a current–time curve produced by the anodic reaction of the metabolically reduced mediator (Fig. 3a). After passing through a minimum due

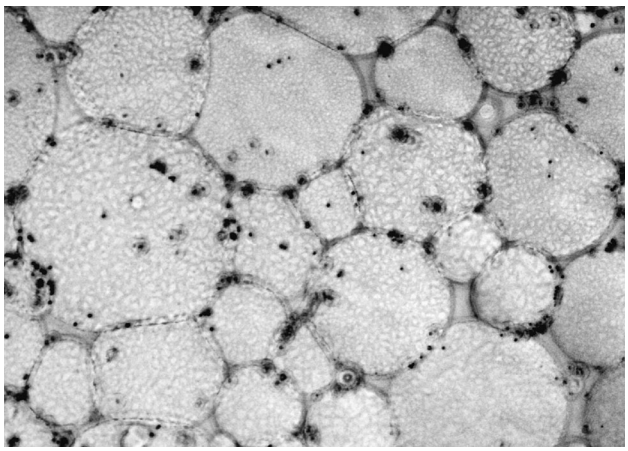

Fig. 2 Pack of swollen SephadexTM globules with cells confined in the interstices (Microscope: OLYMPUS BH2)

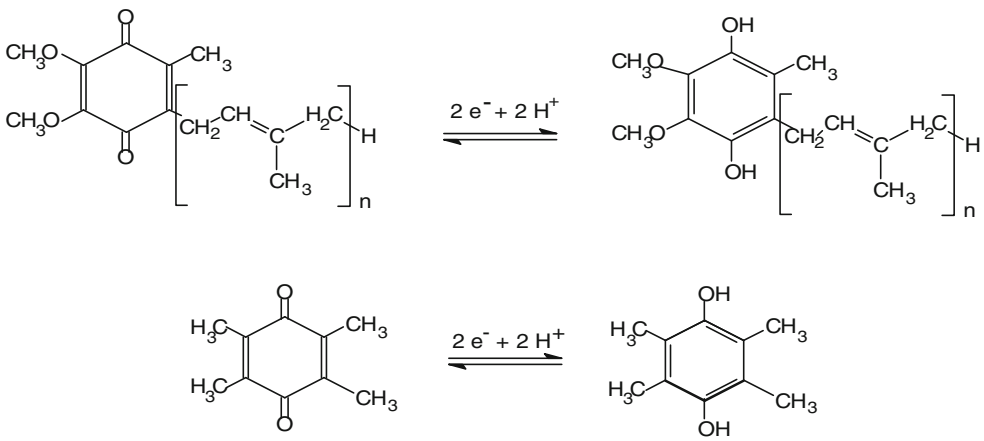

Fig. 1 Comparison of oxidised and reduced coenzyme Q (ubiquinone), *above*, and oxidised and reduced duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone), *below*

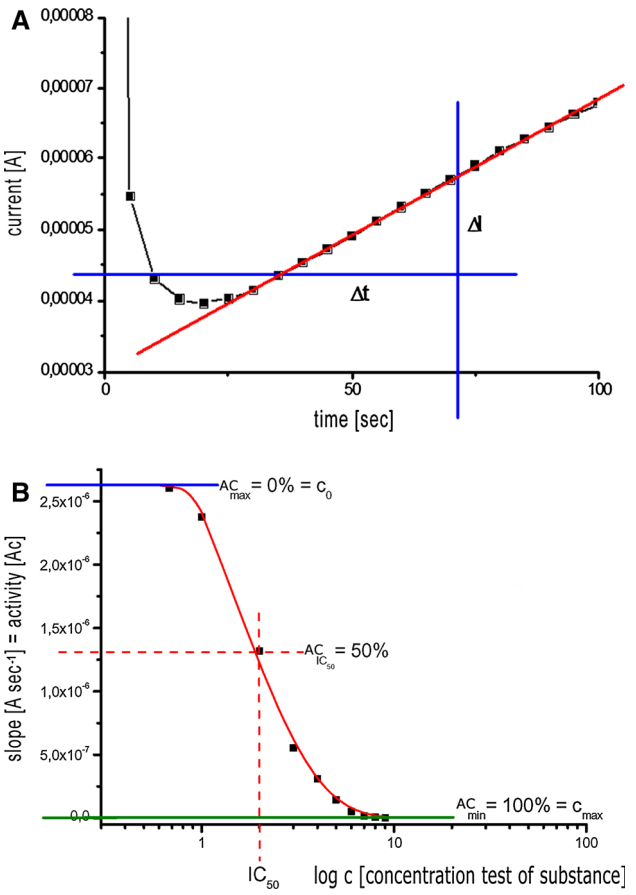

Fig. 3 Evaluation of the data obtained with the novel electrochemical sensor for cytotoxicity. **a** Current/time curve—the slope of the curve is determined by means of the linear portion. **b** Cytotoxicity curve showing the relationship between the slope of the current/time curves (*Y* axis) and the corresponding concentration of the test substance (*X* axis, log scale). ACmax is the value of the maximum cell activity when no test substance is added (negative control); ACmin is the value reflecting the total loss of all cell activity. The middle point of the linear part of this curve (IC50 value) describes the loss of 50 % of cell activity

to an initial current decrease caused by polarisation effects at the electrodes, the course of the electric signal showed a steady increase as a function of the metabolic activity. The slope of the linear part of the current–time curves was used to determine the activity, as it directly describes the rate at which the mediator is reduced by the cells. The linear part of the curve with the greatest positive slope was evaluated; it was related to the concentration of the chemical tested; and the IC_{50} was calculated (Fig. 3b).

2.5 Construction of the measuring cell

The measuring cell used for this study was constructed in house and consisted of a three-electrode system (Fig. 4). The system was designed as a multiple measurement system with four autonomous reaction measurement cells (reaction vessels) allowing the detection of four different measuring points (e.g. four different concentrations of the test substance) simultaneously. The vessels, made of platinum and designed as spherical elements, were used as the counter electrodes. The platinum working electrodes were also designed as spherical elements, but with a smaller surface than that of the counter electrode. The counter electrode and the working electrode had a surface of 160 and 56 mm^2, respectively. During measurements, the counter electrode and the reference electrode interlocked and the interstice between the two electrodes had a working volume of 500 µL. The reference electrode was located at the bottom of the counter electrode and opposite the working electrode.

2.6 Cell lines and culture

The mammalian cell lines Chinese hamster ovary, CHO (ACC 110, German Collection of Microorganisms and Cell Cultures number), Chinese hamster lung fibroblasts V79 (ACC 335) and Swiss albino mouse fibroblasts 3t3 (ACC 173) were used for the electrochemical studies. The reference MTT test studies were performed with the CHO and V79 cell lines. The cells were cultured at 37 °C/5 % CO_2 in 150 cm^2 T-flasks and split 1:4 every 7 days with a periodic change of fresh media. For the measurements, the same number of cells of the same quality (7-day culture) was used.

2.7 Preparation of the cells for measurement

Prior to the measurements, the cell layers from the 150 cm^2 T-flask were washed twice with 5 mL PBS [BIOCHROM, L1820] and dissolved with 1 mL Trypsin/EDTA solution [BIOCHROM T3924]. After cell delamination, the trypsin reaction was stopped with 1 mL media containing 10 % foetal calf serum. Subsequently, the cell suspension was diluted with 2 mL PBS [BIOCHROM, L1820], and the cell suspension from one 150 cm^2 flask was transferred to a 25 cm^2 T-flask. In order to have a quantitative transfer of cells, the 150 cm^2 T-flask was flushed with an additional 2 mL PBS and transferred to the same 25 cm^2 T-flask. Then, the suspension was incubated upright in the flask at 37 °C/5 % CO_2 for 0.5 h. During this time, the cells sedimented on the bottom of the flask. Before starting the tests, 2 mL of the supernatant was discarded to obtain a higher cell concentration in the test suspension.

2.8 Measurement procedure

The stock of Sephadex™/mediator mixture consisted of 50 µmol mediator and 500 mg Sephadex™ G200

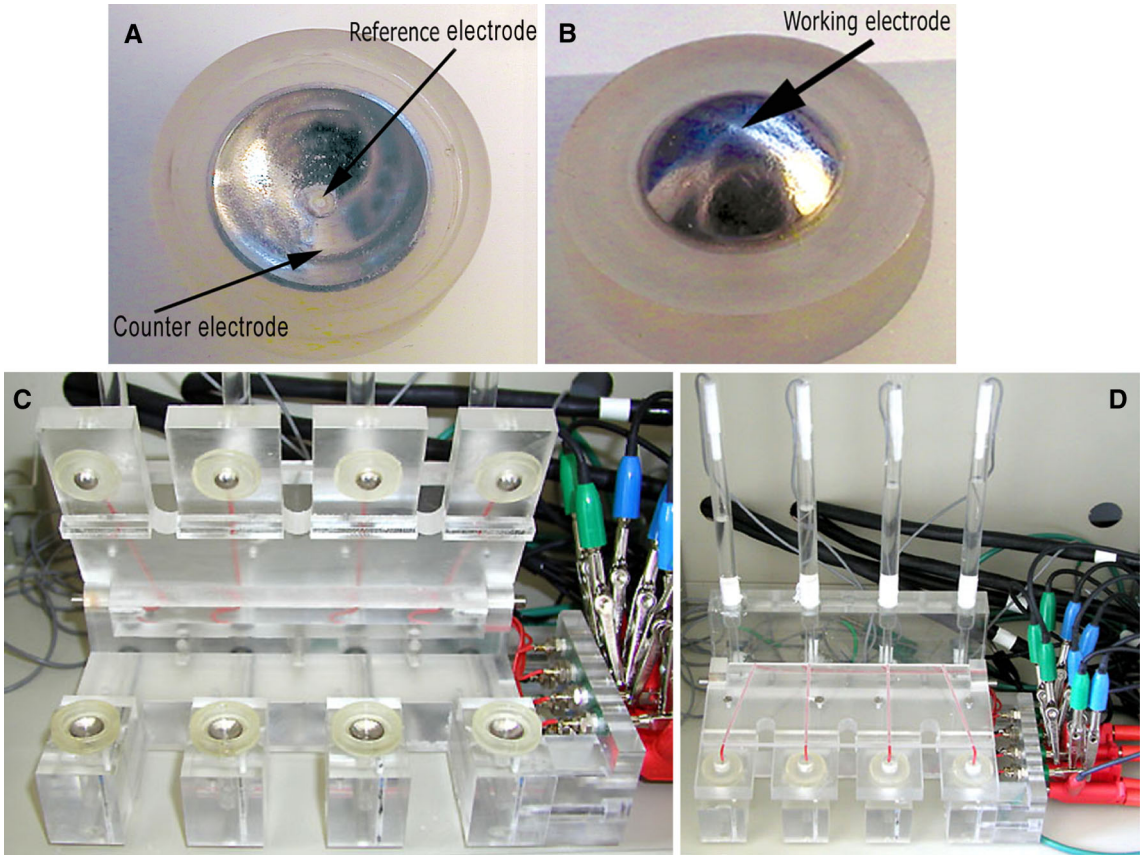

Fig. 4 Reference electrode, counter electrode and overall view of the measuring device. **a** A platinum vessel constructed as a counter electrode. The diaphragm of the reference electrode is integrated into the centre of the counter electrode at the bottom. **b** A platinum working electrode constructed as a spherical element with a smaller diameter than that of the counter electrode. **c**, **d** Open measuring cell with four individual measurement cells, each with a volume of 500 µL

superfine [FLUKA 84961] (depicted in Fig. 5). Before starting the measurement, 25 mg of mediator/Sephadex® mixture was poured into the spherical cap (vessel). The mixture must always be located in almost the same position in the vessel so that the swelling of the mixture is more reproducible. Subsequently, the prepared 500 µL cell suspension was pipetted into the measuring cell. After pipetting the cell suspension onto the mediator/Sephadex™, the gel immediately started to swell. Once it had swollen, the counter electrode fixed in the lid of the measurement cell was introduced into the vessel and potentiostatic data logging commenced (Metrohm PGS-TAD 12, Filterstadt, Germany). At a certain potential, the oxidation of the mediator could be detected and a current–time curve created. Depending on the cells used, the measuring time ranged from 600 to 800 s. In order to ensure the same starting conditions, the measuring cell was flushed with PBS five times before every measurement (Fig. 6).

2.9 MTT test as a reference test method

As a reference method for comparison of the values of the electrochemical measurements, a modified MTT cytotoxicity assay was used. This test is based on the reduction of MTT (3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) to a purple, insoluble MTT formazan by succinate dehydrogenase in intact mitochondria. The measured intensity is proportional to the mitochondrial activity.

The MTT test was performed in 96-well microwell plates. In total, 75 µL of working culture (approx. 10,000 cells, counted by a NEUBAUER chamber after trypan blue staining [BIOCHROM, L6323]) and 75 µL of media containing the test substance (1:1 concentration range) were added to the microplate (only 60 interior wells in the plate were used for the experiments, while the wells at the plate edges were filled with water to ensure constant humidity). The cells were then cultivated under sterile conditions for

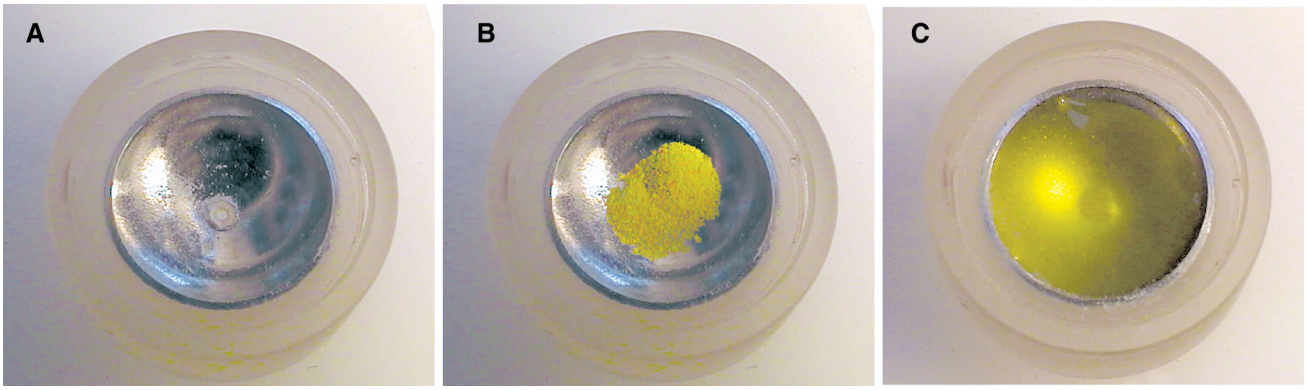

Fig. 5 Preparation of the cell for measurement. **a** Empty measurement vessel (counter electrode) before filling. **b** First step: Measurement cell containing a portion of dry mediator/gel mixture before the sample is added. **c** Second step: The sample is added to the mediator/gel mixture in the measurement vessel to obtain equally swollen SephadexTM globules

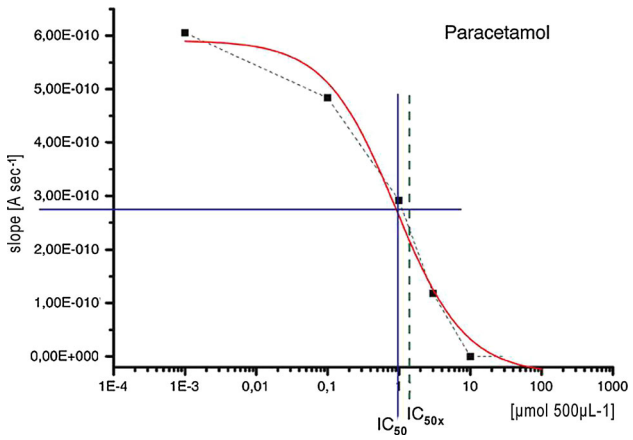

Fig. 6 Cytotoxicity curve of paracetamol created using the values from the electrochemical measurement system. The mediator used was duroquinone. For this test, CHO cells were used as the biological system. The results of the electrochemical test were a good approximation of those from the literature. *Blue line* IC50 of the electrochemical test, *dotted lines* corresponding literature data IC50X (IC50X describes the cytotoxicity of the tested chemical) [48]. (Color figure online)

24 h at 37 °C and 5 % CO_2. After incubation, MTT (0.2 mg/mL) was added for an additional 4 h. The cells were then lysed with 100 µL HCL-isopropanol-SDS solution (while shaken for 15 min), and the absorbance was measured at 590 nm.

3 Results

The electrochemical tests were carried out with three cell lines on eight different substances and compared with the MTT test, and IC_{50}/LD_{50} values found in the literature (Table 1).

For all comparisons, inhibitory concentrations (IC_{50}) were used. The IC_{50} value is a specific value to describe the cytotoxicity of the test substances, which could be read for each test substance from the specific sigmoid cytotoxicity curve calculated. To define the IC_{50} values, test substance concentrations of 0.2, 2, 6, 20 µmol mL^{-1} and blank (0.0 µmol mL^{-1}) were used in our experiments. If necessary, a value for dead cells was also used. In most cases, these increments assured a sufficiently fine gradation of toxicity values to create a complete cytotoxicity curve. The results of IC_{50} were then grouped into four classes—highly toxic, toxic, less toxic and non-toxic—to better present the overall results (see Table 1).

The IC_{50} values derived from the biosensor correlated well with those of the reference MTT tests. The most cytotoxic compounds were lindane and paraquat, which had the lowest IC_{50} (<2 mmol L^{-1}) in both experimental setups compared. Corresponding results were also found for the least toxic chemicals, such as NaCl and isoniazide. In most cases, the rapid electrochemical assay showed better sensitivity than the 24-h MTT test.

4 Discussion

The presented measurement system consists of a pair of electrodes (working and counter electrode) immersed in a gel during the measurement procedure. The advantage of the system demonstrated in the present work is that it documents the activity of micro-organisms or living cells at any given time. The measurements allow a direct assessment of growth, cell viability and cell activity.

A significant advantage of the new measurement system over traditional photometric systems is the test time, which can be as short as 2 h (compared to 24–72 h in standard

Table 1 Comparison of the results of the electrochemical tests with those of the MTT test and data from the literature [47, 48]

	MTT Test IC_{50} [mmol L^{-1}]		Electrochemical Test IC_{50} [mmol L^{-1}]			Literature IC_{50X} [mmol L^{-1}]	Literature LD_{50} Rat [mmol kg^{-1}]	Literature LD_{50} Mouse [mmol kg^{-1}]
	CHO	V79	CHO	3t3	V79			
NaCl	--*	--*	--*	--*	--*	76	51.3	68.4
Isoniazide	10.0	6.2	18.5	11.1	0.7	7.5	4.7	1.0**
Lindane	0.7	0.1	0.1	0.1	0.1	0.4	0.3	0.3
Paracetamol	6.0	3.0	2.0	1.7	2.7	2.7	15.9	2.2
ASS	2.0	3.0	2.1	1.4	1.2	2.3	5.6	4.5
Trichlorfon	3.0	2.7	3.0	0.1	0.1	0.3	1.8	1.2
Cyclohexamide	8.0	1.9	2.4	0.4	1.1	0.6	0.01	0.5
Paraquat	1.8	1.5	1.1	1.4	1.8	0.5	0.3	1.1

Classification

Highly toxic:	0 – 1.9	Toxic :	2.0 – 9.9	Less toxic :	10-99	Non-toxic:	> 99

* Due to the limited volume of the vessel, it was not possible to add an adequate amount of NaCl to the chamber of the measurement cells to determine the cytotoxic effects of NaCl and thus to generate a complete cytotoxic curve

** No value available in the literature consulted for this study [48]. For this value we used a material safety data sheet (MSDS) [47]

assays). The calculated IC_{50} values were classified into four groups—non-toxic, less toxic, toxic and highly toxic—which rapidly provided an overview of the cytotoxicity of the test chemical concerned.

A further advantage of the proposed electrochemical measurement system is the use of powdery lipophilic test substances without any preliminary steps. This precludes, for example, the need to dissolve lipophilic substances in acetone or DMSO, which may cause unwanted side reactions or additional toxic effects in the cells. The measurement system can be used not only for toxicological tests, but also in pharmaceutical screening to identify the effectiveness of chemical substances. Furthermore, the test system could also be used in the field of environmental toxicology using conventional model organisms such as algae and/or cyanobacteria, for which the mediator has already been described [49]. Further research could also investigate the application of such an electrochemical measurement system when used with a mixture of different cells, micro-organisms or cell assemblages, such as animal tissues.

To facilitate the suggested approaches, further work is needed to upscale the electrochemical cell to a standard microwell plate format. This would allow easy handling, high throughput and rapid derivation of a complete cytotoxic dose–response curve in just a single measurement step.

5 Conclusion

In summary, the functionality of the multi-cell developed for cytotoxicity testing in mammalian cell cultures was demonstrated. The electrochemical assay was able to provide accurate information on the cytotoxicity of a series of tested substances, thus demonstrating its viability for use as a toxicological in vitro screening tool. The system is easy to use, and the measurement time is significantly reduced. Furthermore, scale-up of the system to a microtiter plate format would enable full automation and high-throughput applications for the chemical, life science and pharmaceutical industries.

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